Environmental Health Perspectives
Vol. 76, pp. 33-39, 1987

Mutations and Homologous Recombination Induced in Mammalian Cells by Metabolites of Benzo[a]pyrene and 1-Nitropyrene

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Metabolites of two structurally related chemical carcinogens, benzo[a]pyrene and 1-nitropyrene, were compared for their ability to cause cytotoxicity and induce mutations in normally repairing or nucleotide excision repair-deficient diploid human fibroblasts; for their ability to induce mutations in a defined gene sequence, *supF*, when a plasmid containing adducts formed by these carcinogens replicates in human 293 cells; and for their ability to induce homologous recombination between duplicated genes in mouse L cells. Both of the metabolites tested, i.e., (±)-7β,8α-dihydroxy-9α,10α, epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) and 1-nitrosopyrene (1-NOP), form adducts on guanine. BPDE binds principally at the N2 position of guanine; 1-NOP binds to guanine at the C8 position. Results of the studies in diploid human cells indicated that when compared on the basis of equal numbers of DNA adducts, BPDE is more effective than 1-NOP in inducing mutations in DNA repair-proficient cells, but when compared in repair-deficient xeroderma pigmentosum human cells that do not remove such adducts from their DNA, the frequency of mutants induced per adduct is equal. These results suggest that during the time available for repair of potentially mutagenic lesions, repair-proficient human cells excise 1-NOP adducts more rapidly than they excise BPDE adducts. Molecular analysis of the specific kinds of mutations induced when a plasmid containing BPDE residues was allowed to replicate in human cells showed that BPDE induces mainly base substitution mutations, predominantly G:C to T:A transversions. Preliminary results with 1-NOP-treated plasmids in that system indicate that 1-NOP is not as mutagenic as BPDE when the two carcinogens are compared on the basis of equal initial numbers of adducts per plasmid. Just as was the case for mutation induction in the genome of diploid human fibroblasts discussed above, the difference in mutagenicity per adduct may reflect a difference in excision repair rate by the human cell line 293. This question is under investigation. In addition, preliminary results of a study comparing 1-NOP with BPDE for ability to induce homologous recombination in mouse L cells indicate that BPDE is more effective than 1-NOP.

Introduction

As a basis for risk assessment, it is important to determine the biological effects of exposure of animals and human beings to environmental carcinogenic and mutagenic agents. Our laboratory has been investigating the biological effects of such agents in mammalian cells in culture in an effort to understand the mechanisms of mutagenesis and carcinogenesis at the cellular and molecular level. The majority of our studies have been carried out with diploid human fibroblasts, but more recently we have also employed an established human cell line transformed by DNA from adenovirus and a thymidine kinase-deficient mouse L cell line.

As part of studies of the mechanisms of carcinogenesis, we and our co-workers have investigated the cytotoxicity, mutagenicity, and transforming activity of a number of chemical carcinogens and radiation in diploid human fibroblasts that differ in their ability to excise DNA lesions caused by such agents. For example, we have asked whether cell killing and/or mutation induction is correlated with the initial number of DNA adducts or the number of adducts remaining at a particular time posttreatment; whether potentially mutagenic lesions are converted into mutations during excision repair, the role of semiconservative DNA replication in mutation induction; whether particular lesions or adducts are intrinsically more mutagenic or more cytotoxic than others; and whether genetic changes resulting from unrepaired DNA lesions are causally involved in the process of neoplastic transformation.

To address these questions, we developed quantitative assays for measuring the cytotoxic, mutagenic, and transforming effect of carcinogens in diploid human skin

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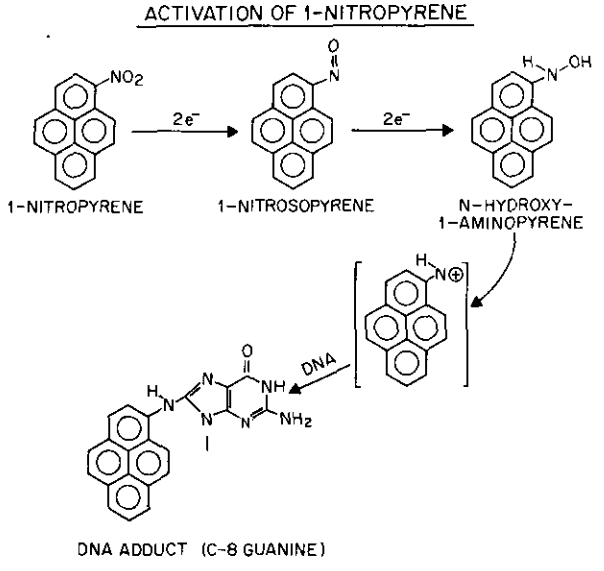

FIGURE 1. Proposed pathway for cellular activation of 1-NP. Metabolism proceeds via reduction of the parent compound to 1-NOP, followed by reduction to the corresponding hydroxylamine and the formation of a nitrenium intermediate which reacts with DNA to form a covalently bound adduct, i.e., *N*-(deoxyguanosin-8-yl)-1-aminopyrene. After Patton et al. (*11*) with permission.

fibroblasts derived from neonatal foreskin or skin biopsy material (*1–4*). Human cells grow well in culture and form colonies at low cell density with an efficiency of 40 to 80%. They can be treated with direct-acting mutagens and the cytotoxic effect quantitated from the decrease in their cloning ability. Compounds requiring metabolic activation can also be used if the cells are provided with a source of activation, such as metabolizing human feeder cells (*5,6*). The dose-dependent induction of mutations by carcinogens can be quantitated by determining the frequency of cells able to grow in the presence of 6-thioguanine (TG) as a result of mutations that inactivate hypoxanthine(guanine)phosphoribosyltransferase (*1–3*) or in the presence of diphtheria toxin (DT) as a result of inactivation of its receptor site on the elongation factor 2 (*7*). The frequency of transformation of normal diploid human cells can be measured by determining the frequency of cells able to exhibit anchorage-independent growth, i.e., colony formation in semisolid medium containing 0.33% agar (*3,4*). Such anchorage-independent diploid human cells still possess a finite life-span in culture and are not capable of producing malignant tumors, but anchorage independence is a phenotype highly characteristic of tumor-derived cells.

For studies on the effect of DNA repair on these processes, we have made use of nucleotide excision repair-deficient fibroblasts derived from xeroderma pigmentosum (XP) patients (*2,3*). In particular, we have studied XP12BE cells, which we showed are unable to excise UV radiation-induced photoproducts from DNA or multiringed residues formed in DNA by reactive derivatives of polycyclic aromatic carcinogens, such as 2-acetylaminofluorene (*8*), benzo[a]pyrene (*9,10*), and 1-nitropyrene (*11,12*). We have recently extended our studies of the biological effects of carcinogens and mutagens to determine, at the sequence level, the number and kinds of mutations induced in a defined gene, *supF*, when carcinogen-treated plasmid is replicated in an established human cell line (*13*). We have also begun to assay the ability of such agents to induce homologous recombination between duplicated genes in the genome of mouse L cells (*14*). In this report we summarize the data we have obtained using metabolites of two structurally related polycyclic aromatic carcinogens, benzo[a]pyrene and 1-nitropyrene. The metabolites studied are (±)7-β,8α-dihydroxy-9α,10α,epoxy-7,8,9,10-tetrahydrobenzo[a]pyrene (BPDE) and 1-nitrosopyrene (1-NOP), the partially reduced metabolite of 1-nitropyrene. Both metabolites are carcinogenic and both bind covalently to guanine. 1-NOP forms only a single major DNA adduct at the C8 position (Fig. 1) (*15*); BPDE forms its principal adduct at the N2 position of guanine (*16*).

Comparison of the Cytotoxic and Mutagenic Effects of BPDE and 1-NOP in Diploid Human Cells

Cytotoxicity and Mutagenicity of BPDE in Diploid Human Fibroblasts

Normally repairing foreskin-derived diploid human fibroblasts and nucleotide excision repair-deficient cells derived from XP patient XP12BE were compared for their sensitivity to the cytotoxic and mutagenic effects of BPDE as a function of the applied concentration. Normal cells in exponential growth were trypsinized, plated at densities of 3.3 to 8 × 10^3 cells/cm^2 in culture medium containing serum, and allowed to attach for 16 hr. The medium was exchanged for serum-free medium and the carcinogen, freshly dissolved in anhydrous dimethylsulfoxide or acetone, was introduced into the dishes by micropipette as described previously (*10*). In the case of the XP cells, the same conditions were employed for most of the experiments, but in one, the cells were treated at confluence, as described (*10*). The number of target cells was adjusted so as to have at least 10^6 surviving cells for each determination. Cytotoxicity was determined by immediately plating cells at low (cloning) densities, i.e., 50 to 500 cells per dish depending on the expected number of colonies. A series of dishes containing cells plated at various low densities was employed so as to have six or more dishes containing ~40 colonies each. Mutagenicity was assayed by keeping treated and untreated populations in exponential growth during a 7 to 10 day expression period before selecting 1 to 2 × 10^6 cells per determination for the frequency of cells resistant to 6-thioguanine (*1,7,11*).

The results are shown in Figure 2. The repair-deficient XP cells were significantly more sensitive than the normal cells to the cytotoxic and the mutagenic effects

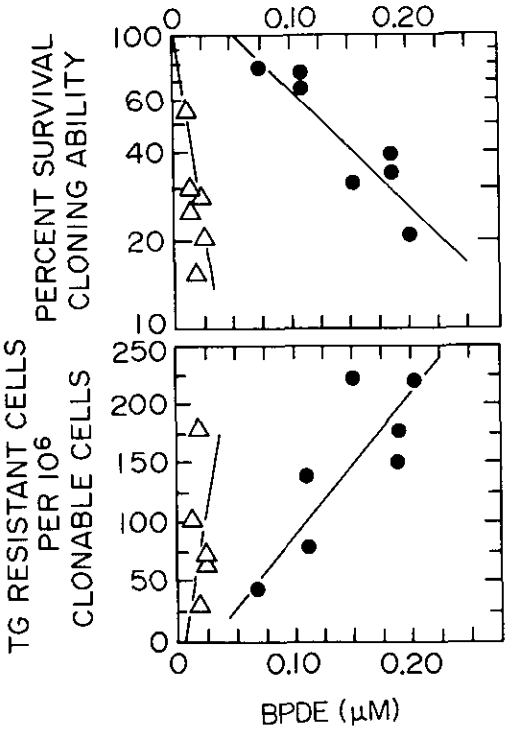

FIGURE 2. Cytotoxic and mutagenic effect of BPDE in normal (•) or XP (△) human fibroblasts as a function of concentration. The cells were treated at a density of 0.33–0.8 × 10^4 cells/cm^2. The background mutant frequencies per 10^6 clonable cells ranged from 0 to 22 for XP cells and from 5 to 19 for normal cells. The mutagenicity data have been corrected for these backgrounds. The straight line portions of the curves were calculated by the method of least squares.

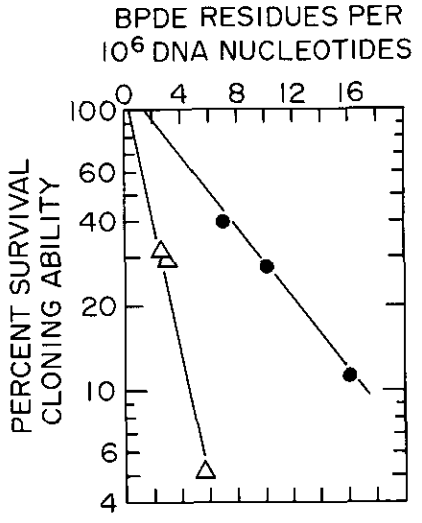

FIGURE 3. Comparison of the cytotoxic effects of BPDE in normal (•) or repair-deficient diploid human cells (△) as a function of the initial number of residues bound to cellular DNA at the end of the exposure period. The cells were treated for 1 hr at confluence with tritiated BPDE (XP, 0.025 and 0.05 μM; NF, 0.15, 0.2, and 0.3 μM) and then immediately harvested and assayed for survival and extent of binding to cellular DNA. Lines were determined by the method of least squares. The majority of these data were taken from (*9*).

of BPDE. A concentration of 0.02 μM was sufficient to lower their survival to 37% of the untreated control; 0.16 μM was required for the repair-proficient normal cells. Those same concentrations increased the frequency of mutations to 75 × 10^{-6} and 160 × 10^{-6}, respectively.

Evidence that the abnormal sensitivity of the XP cells to BPDE reflected an inability of the cells to remove BPDE adducts from their DNA was published previously (*9,10*). Such a conclusion can be drawn from the data in Figure 3, showing that XP12BE cells initially possessing six BPDE residues per 10^6 DNA nucleotides exhibit a survival of 5%, but normally repairing cells with the same initial number of adducts exhibit a survival of 50%. It takes eight initial residues per 10^6 nucleotides to lower the survival of the normal cells to 37%, but only two per 10^6 nucleotides for the XP cells, suggesting that the repair-proficient cells remove many of the potentially cytotoxic residues from DNA before these can exert their killing effect.

Cytotoxicity and Mutagenicity of 1-NOP in Diploid Human Fibroblasts

A similar comparative study was carried out by Patton et al. (*11,12*) using 1-nitropyrene and 1-nitrosopyrene. The results with 1-NOP are shown in Figure 4. Again, the repair-deficient XP cells proved significantly more sensitive than normal cells to the cytotoxic and mutagenic effects of this multiringed carcinogen. A concentration of 0.16 μM reduced their survival to 37% of the control. For the repair-proficient normal cells 0.6 μM was required. Those same concentrations increased the frequency of mutations to 150 × 10^{-6} and 70 × 10^{-6}, respectively.

Evidence that the abnormal sensitivity of the XP cells to 1-NOP reflected their inability to remove adducts from their DNA can be seen in Figure 5. Given approximately the same concentration of 1-NOP, i.e., 0.7 and 0.73 μM, normal and XP cells exhibited the same initial number of DNA adducts per 10^6 nucleotides (i.e., ~22). But XP cells exhibited a survival of 0.2% and normal cells showed 25% survival. It takes 25 initial 1-NOP residues to lower the survival or the normal cells to 37%, but only four per 10^6 nucleotides for the XP cells. In contrast to what we found with BPDE, at a 37% survival dose, the normal cells have a frequency of induced mutations twofold lower than the XP cells, suggesting that the normal cells were able to remove potentially mutagenic 1-NOP adducts from their DNA before these adducts could exert their mutagenic effect. This difference in the mutation results obtained with the two compounds suggests that normal human cells remove 1-NOP-induced residues from their DNA faster than they remove BPDE adducts.

Because of these possible differences in rate of excision repair of adducts, it is not as informative to compare the mutagenic effect of two agents on the basis of the

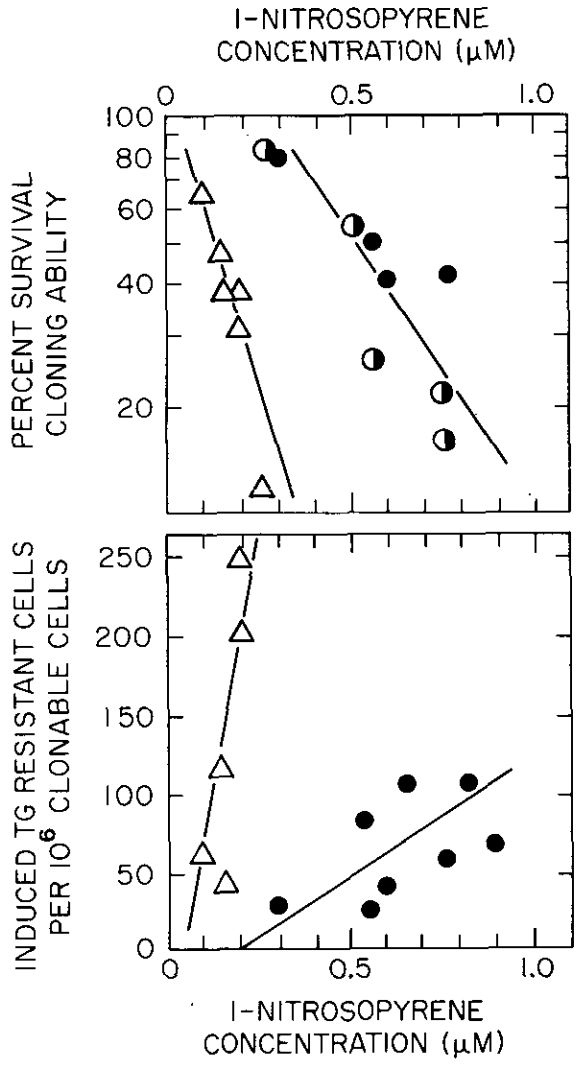

FIGURE 4. Cytotoxic and mutagenic effect of 1-NOP in normal (•) or XP (△) human fibroblasts as a function of concentration. The cells were treated at a density of 0.33–1 × 10^4 cells per cm^2. The background mutant frequencies per 10^6 clonable cells ranged from 0 to 13 for XP cells and from 0 to 8 for normal cells. The mutagenicity data have been corrected for these backgrounds. The straight line portions of the curves were calculated by the method of least squares. After Patton et al. (*11*) with permission.

initial number of DNA adducts formed. However, if one is working with a cell line that does not remove adducts, e.g., XP12BE cells, then the number of adducts remaining in the DNA at the time potentially mutagenic lesions are converted into mutations ("fixed") is the same as the initial number of adducts. Therefore, one can validly compare the frequency of mutants induced in such cells as a function of initial adducts. A comparison of the data in Figures 2 through 5 shows that in XP12BE cells, two BPDE adducts per 10^6 nucleotides induces a mutant frequency of ~75 × 10^{-6}, whereas four 1-NOP adducts per 10^6 nucleotides induces ~150 × 10^{-6}. Thus, the mutagenic effectiveness of the agents in these cells appears to be equal.

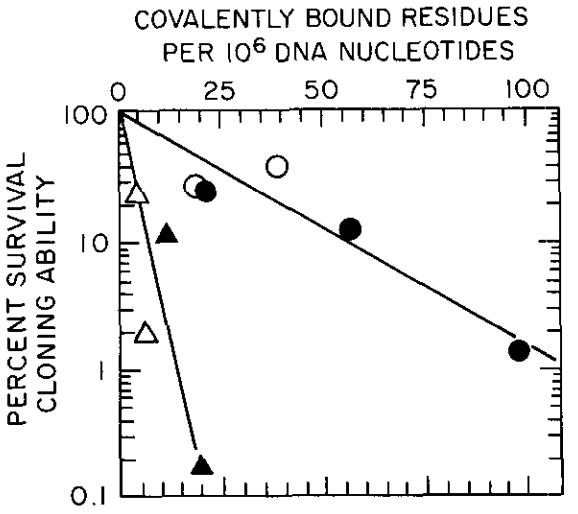

FIGURE 5. Comparison of the cytotoxic effects of 1-NP (open symbols) and 1-NOP (closed symbols) in NF (•, ○) and XP (▲, △) cells as a function of the number of residues bound to cellular DNA at the end of the exposure period (1-NP, 10 hr; 1-NOP, 1 hr). Exponentially growing cells at a density of 10^4 cells/cm^2 were exposed to radioactive-labeled compound (1-NP; XP, 14 or 20 μM; NF 50 and 80 μM; 1-NOP; XP 0.36 and 0.73 μM; NF, 0.7, 1.0 and 2.1 μM). The cells were harvested and assayed for survival and extent of binding to cellular DNA. Lines were determined by the method of least squares. After Patton et al. (*11*) with permission.

Comparison of the Mutagenicity of BPDE and 1-NOP Adducts When a Plasmid Replicates in a Human Cell Line

Number and Kinds of Mutations Induced by BPDE

Recently, Yang et al. (*13*), using a shuttle vector, pZ189, showed that when the plasmid carrying BPDE adducts replicated in human 293 cells, the mutations induced in the target gene, *supF*, coding for a tyrosine tRNA, were predominantly single base substitutions. The frequency of mutants induced in this system as a function of BPDE adducts is shown in Figure 6. DNA sequence analysis of 86 independent mutants derived with BPDE-treated plasmids showed that the majority (60/86) exhibited base substitutions, with the majority (75%) being G:C to T:A transversions (*13*). Two very prominent hot spots were found. Molecular analysis of 30 spontaneous mutants showed that the majority (70%) involved deletions, insertions, or altered gel mobility.

Preliminary Studies with 1-NOP

These same investigators are currently undertaking a similar study using the pZ189 plasmid treated with tritiated 1-NOP in the presence of ascorbic acid. Preliminary results suggest that when compared on the basis of equal numbers of adducts per plasmid, 1-NOP is not as effective as BPDE in inducing mutations when

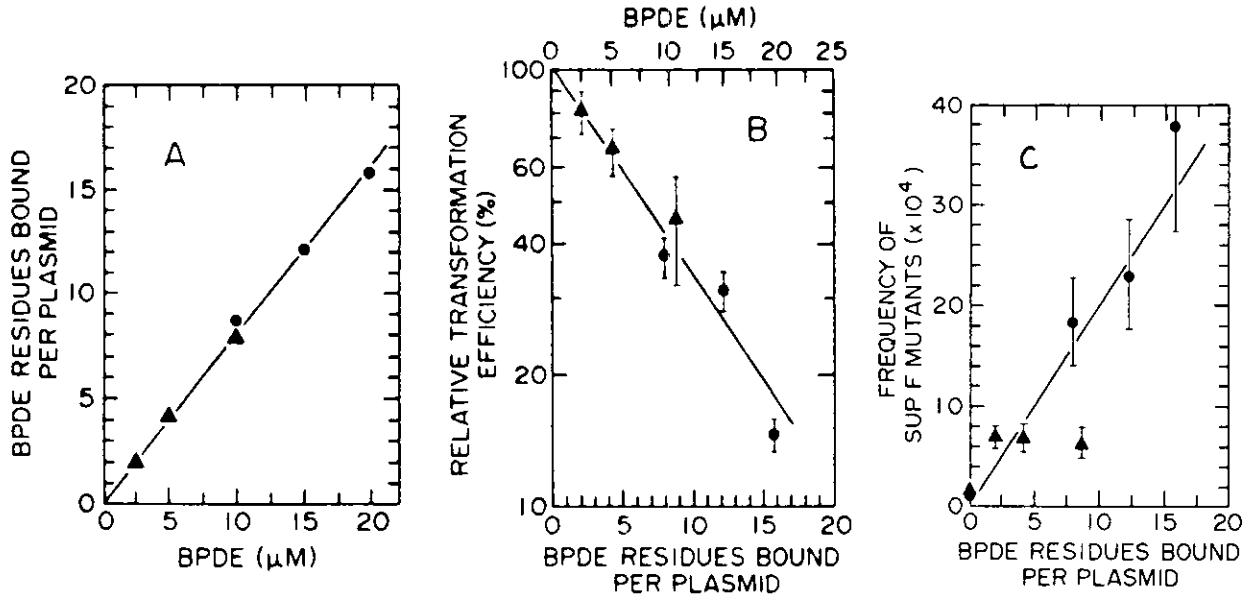

FIGURE 6. (*A*) BPDE residues per pZ189 plasmid as a function of applied concentration; (●) and (▲) denote two separate binding experiments. (*B*) Frequency of transformation of the *E. coli* host to ampicillin resistance by BPDE-modified plasmid. The error bars indicate the SEM of five determinations. (*C*) Frequency of *supF* mutants induced when the BPDE-treated plasmid replicated in human cells 293 cells, plotted as a function of the number of BPDE residues per plasmid. The error bars refer to SEM of the mutant frequencies from a series of human cell transfection experiments made with each set of treated plasmid. Taken from (*13*) with permission.

the plasmid replicates in human cells. Whether this difference reflects the intrinsic difference in the nature of the guanine adducts formed or reflects a difference in the rate of removal of the adducts from the plasmid by the human 293 cell line before they can cause mutations is currently under investigation in our laboratory.

Comparison of the Recombinagenic Effect of BPDE and 1-NOP in Mouse L Cells

Frequency of Recombination Induced by BPDE

To try to understand the mechanisms of chemical and physical carcinogenesis, we have investigated whether carcinogens can induce homologous recombination between genes located within the mammalian cell genome and characterized the kinds of recombination events induced (*14*). A comparison of the various agents for their ability to induce recombination as a function of their cytotoxic effect or the number of DNA lesions they induce could shed light on the mechanisms involved. The system employed was designed by Liskay et al. (*17*). It involves a thymidine kinase (tk)-deficient mouse L cell line, designated 333M, which contains a single, stably integrated plasmid carrying duplicated copies of the Herpes simplex virus *tk* gene (H*tk*), each inactivated by an eight bp *Xho* I linker mutation at a unique site. After exposure to the mutagenic agents, the tk^- target cells are selected for expression of a functional H*tk* enzyme, which requires a productive recombinational event between the two nonfunctional genes. To date, the mutagens we have tested in this system include ultraviolet radiation, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, mitomycin C, and BPDE (*14*).

For such studies, sufficient target cells are used so as to have at least 2×10^6 surviving cells to be assayed for recombination frequency by selection in medium containing cytidine, hypoxanthine, aminopterin, and thymidine (CHAT medium) (*14*). The results obtained with BPDE are shown in Figure 7. The doses required to reduce the cell survival to between 90% and 10% are very similar to those for normal diploid human fibroblasts (Fig. 2). There was a dose-dependent increase in the frequency of tk^+ recombinants. The highest doses (2.5 μM) increased the frequency from a background frequency of 19×10^{-6} to 60×10^{-6}, giving an induced frequency of 41×10^{-6}. A 37% survival dose (0.18 μM) gave an induced frequency of 25×10^{-6}. Binding data with radiolabeled BPDE showed that at 37% survival the number of adducts is 9 per 10^6 DNA nucleotides (data not shown).

Frequency of Recombination Induced by 1-NOP

Results of preliminary studies with this system using 1-NOP showed that the concentration needed to reduce the survival of mouse L cells to 37% was 0.6 μM (data not shown). This quantity is similar to that needed for

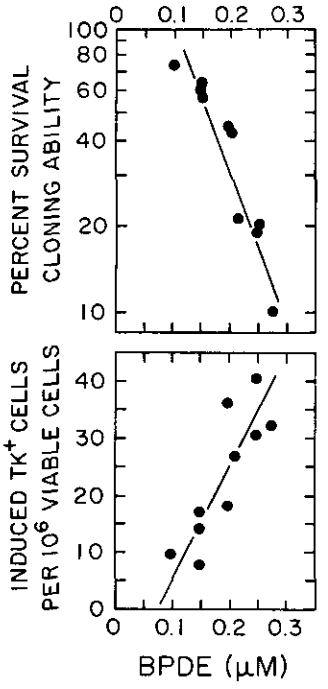

FIGURE 7. Cell killing and induction of recombination as a function of the concentration of BPDE. The cells were plated at 10^4 cells/cm^2 and treated with BPDE for 1 hr. Immediately following treatment, one set of cells were plated at cloning densities to assay survival. The rest were allowed ~20 hr before being selected *in situ* for resistance to CHAT medium (*tk*$^+$ recombinants). The background frequencies in the three experiments ranged from 16.3×10^{-6} to 19.5×10^{-6}. These have been subtracted to give induced frequences of recombinants. Taken from (*14*) with permission.

normal diploid human cells, i.e., 0.75 μM (Fig. 4). The frequency of tk$^+$ recombinants induced by 1-NOP at that concentration was 32×10^{-4} (data not shown). In human fibroblasts, an applied concentration of 0.7 μM 1-NOP induced 23 adducts per 10^6 nucleotides (Fig. 5). Binding data with radiolabeled 1-NOP showed that at 37% survival (0.6 μM) the number of adducts is 34 per 10^6 DNA nucleotides. Therefore, the recombinagenic activity of 1-NOP per adduct in mouse L cells is at least twofold lower than that of BPDE. However, before drawing conclusions about the ability of DNA adducts to induce such biological effects, it will be necessary to determine if mouse L cells can excise these adducts from their DNA and whether they remove 1-NOP adducts faster than they excise BPDE residues. Such comparative studies are presently underway in our laboratory.

Kinds of Recombination Events Induced by These Agents

The plasmid used by Liskay et al. (*17*) to transfect mouse L cells to obtain the 333M target cells was designed to facilitate analysis of the kinds of recombination events which take place. In addition to duplicate H*tk* genes, each with an eight bp *Xho* I linker frameshift mutation in a unique location, the plasmid contains the *neo* gene coding for Geneticin resistance located between the two H*tk* genes to facilitate assaying the *tk*$^+$ recombinants for the retention or loss of the intervening DNA sequence. If the recombination event involves a single reciprocal exchange within a chromatid or a single unequal exchange between sister chromatids, only a single wild-type copy of the H*tk* cell gene will be present and the sequence containing the *neo* gene will be lost. In contrast, if the recombination event consists of a nonreciprocal transfer of wild-type information (gene conversion), the H*tk* gene duplication, including the *neo* gene, will be retained in the recombinant, but one H*tk* gene will be wild-type. Therefore, simply assaying *tk*$^+$ recombinants for resistance to Geneticin can distinguish which type of event occurred. Southern blot analysis of DNA from such recombinants can readily be used to confirm these results, since each H*tk* gene was inserted at a unique restriction site locus in the original plasmid (*17*). Wang et al. (*14*) have carried out such a study and found that 85 to 90% of the BPDE-induced recombinants involved gene conversion. Thus, BPDE raised the frequency of both kinds of recombinant events in the same proportions as they occur spontaneously (*17*). Investigation of the kinds of events induced by 1-NOP has not yet been carried out.

Conclusion

Our studies with BPDE and 1-NOP as model environmental carcinogens in excision repair-proficient and repair-deficient human fibroblasts indicate that when the agents are compared on the basis of applied concentration, BPDE is more active than 1-NOP. When the mutagenicity of two agents is compared at doses causing equal cytotoxicity ("mutagenic efficiency") in the repair-proficient cells, BPDE is more mutagenic than 1-NOP. This is also true when the two are compared for the frequency of mutants induced in normal human cells as a function of the initial number of DNA adducts formed ("mutagenic effectiveness"). However, in XP cells that are unable to remove BPDE- or 1-NOP-induced adducts, the mutagenic effectiveness of the two carcinogens is equal. This suggests that following exposure to the agents, the repair-proficient cells remove a greater fraction of the potentially mutagenic 1-NOP-induced adducts than BPDE-induced adducts before these can be converted into mutations. This prediction would explain why plasmids containing BPDE-induced adducts showed a higher frequency of mutants induced in the target gene (*supF*) than did plasmids carrying 1-NOP adducts.

The ongoing study comparing BPDE and 1-NOP for their recombinagenic ability in mouse L cells suggests that BPDE is twice as effective as 1-NOP. However, additional studies are required before one can draw this conclusion since the mouse L cells also may be capable of removing 1-NOP-induced mutants faster than BPDE-induced adducts. Nevertheless, the data from each of the studies described here demonstrate the importance

of determining the biologic effect of given agents not only on the basis of applied concentrations, but also as a function of DNA damage, both initial and persistent.

We thank R. Michael Liskay, Yale University School of Medicine, for providing us with the mouse L cell line 333M, Michael Seidman, Otsuka Pharmaceutical Co., for providing us with the pZ189, and Michele Calos, Stanford University, for providing the 293 cell line used for these studies. The research was supported in part by DHHS Grant CA21253 from the National Institutes of Health and by Contracts 83-22 and 87-2 from the Health Effects Institute for the study of 1-nitropyrene and 1-nitrosopyrene. The research conducted under contract to the Health Effects Institute (HEI), an organization jointly funded by the United States Environmental Protection Agency (EPA) (Assistance Agreement X-812059) and automotive manufacturers, is currently under review by the Institute. The contents of this article do not necessarily reflect the views of the HEI, nor do they necessarily reflect the policies of EPA or automotive manufacturers.

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